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Presence of a Virulence-Associated Plasmid in *Yersinia pseudotuberculosis*

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We have shown that *Yersinia pseudotuberculosis* can possess plasmids which are similar in size and function to the previously described Vwa plasmids of *Y. enterocolitica*. These plasmids are associated with the production of V and W antigens (calcium dependency) and pathogenicity of the organism. Further investigation of these plasmids from *Y. pseudotuberculosis* and *Y. enterocolitica* with restriction endonucleases revealed significant differences in their fragmentation pattern.

It has long been recognized that *Yersinia pseudotuberculosis* and *Yersinia pestis* share a variety of physiological, biochemical, serological, and virulence properties (3). From recent studies, it has become apparent that the third member of this genus, *Yersinia enterocolitica*, also can exhibit properties previously associated with *Y. pestis* and *Y. pseudotuberculosis*. In addition to some deoxyribonucleic acid (DNA) relatedness and other taxonomic similarities (2, 10), *Y. enterocolitica* has been shown to possess virulence antigens of plague bacilli. An important factor related to the virulence of *Y. pestis* and *Y. pseudotuberculosis* is their ability to produce a protein-lipoprotein complex, termed the V and W antigen (4, 8). Although the precise role of these components in pathogenesis is still obscure, there is evidence to suggest that expression of the V and W antigen complex may (i) provide the pathogen with a resistance to phagocytosis and (ii) allow intracellular multiplication of the organism within macrophages (3). It has been shown recently that certain strains of *Y. enterocolitica* also produce the V and W antigens, their production being demonstrated both serologically (P. B. Carter, R. J. Zahorchak, and R. R. Brubaker, personal communication) and physiologically as an expression of calcium dependency (6). The calcium dependency of virulent *Y. enterocolitica* strains was shown by inhibiting cell growth at 37°C on magnesium oxalate agar (6). This medium, which is deficient in free calcium, had been previously found to inhibit growth of *Y. pestis* and *Y. pseudotuberculosis* strains which express the V and W antigens (7). Strains unable to produce these antigens are not inhibited and grow well at 37°C on magnesium oxalate agar. Recent studies have established that some virulence properties of *Y. enterocolitica* are possibly plasmid mediated (6, 16). Our recent studies have now shown that *Y. enterocolitica* strains can possess a group of plasmids, which we have designated Vwa, that are associated with the calcium dependency (production of V and W antigens) and pathogenicity of this organism (6).

Y. enterocolitica has gained attention as a significant cause of gastroenteritis which is characterized by diarrhea and invasive involvement of the mesenteric lymphatics (1, 14). Although *Y. pseudotuberculosis* is associated classically with fatal, acute, septicemic infections of rodents, it is also recognized that this pathogen can cause enteric disease in humans, characterized by diarrhea, mesenteric lymphadenopathy, and symptoms of appendicitis (5). It is evident, therefore, that these clinical manifestations of infections by *Y. pseudotuberculosis* can in some circumstances be similar to those described for acute gastroenteritis caused by *Y. enterocolitica*. A consideration of these similarities as well as the knowledge that both species share the V and W virulence antigens has led to the present examination of the plasmid content of a virulent, invasive strain of *Y. pseudotuberculosis*.

The *Y. pseudotuberculosis* serotype III strain and the two *Y. enterocolitica* serotype O:8 strains that we employed were kindly provided by Ira Melman, Food and Drug Administration, Washington, D.C. Our characterization of *Y. pseudotuberculosis* strain III revealed this strain to be lytically sensitive to rough specific phages Ffm and BR 2 (13). Despite this apparent roughness, this strain was highly invasive when we employed the Sereny test for keratoconjunctivitis (12) as a virulence model. After a drop of a broth suspension of strain III (~10^{10} cells per

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ml) was deposited into the conjunctival sac of guinea pigs, keratoconjunctivitis readily developed within 48 to 72 h. Such infected guinea pigs frequently died within 5 days of challenge. Invasion and extraintestinal translocation of strain III within the guinea pig host could easily be documented by recovery of the challenge organism from exudates of diseased eyes and from samples of liver and spleen taken at necropsy.

The plasmid content of strain III was next examined. A clone of strain III, reisolated from the exudate of an infected guinea pig eye, was used for plasmid DNA isolation. Purified covalently closed circular plasmid DNA was recovered from Triton-X cell lysates of strain III by means of cesium chloride-ethidium bromide density gradient centrifugation (6) and examined by means of agarose gel electrophoresis (9). Figure 1 represents a comparison of the plasmid profile of *Y. pseudotuberculosis* strain III with that of *Y. enterocolitica* strain Y7P. The virulence and plasmid properties of *Y. enterocolitica* strain Y7P have been previously described in detail (6). This strain is invasive and provokes both keratoconjunctivitis in guinea pigs and a lethal sepsis in mice. Strain Y7P possesses two plasmids, pWR42 and pWR36, which are 42 and 36 megadaltons (Mdal) in size, respectively. pWR42 has Vwa-associated functions, whereas pWR36 remains cryptic in its function. Loss of the pWR42 plasmid results in loss of the virulence properties of the strain (6). It is evident that *Y. pseudotuberculosis* strain III (Fig. 1). contains a plasmid of about 42 Mdal, a size similar to pWR42 of Y7P. We have designated this plasmid of *Y. pseudotuberculosis* as pWR43. Included as additional size references in this experiment were pWR53, a plasmid of 60 Mdal which controls sucrose fermentation (15) and plasmid DNA from an avirulent Y7P derivative which lacks pWR42, but retains the 36-Mdal cryptic plasmid pWR36. Since the DNAs from Y7P and its avirulent derivative (slots C and D) were only partially purified by an ethanol-precipitated preparation (9), significant contamination with fragmented chromosomal DNA is present. Only a faint amount of such chromosomal DNA contamination is evident in the dye-buoyant density gradient purified preparations (slots A and B).

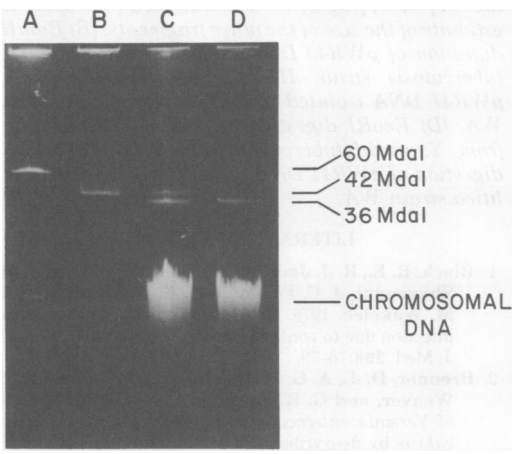

FIG. 1. *Comparison of plasmid DNA from Y. pseudotuberculosis and Y. enterocolitica strains. DNA samples were electrophoresed in 0.7% agarose gels dissolved in tris(hydroxymethyl)aminomethane-borate buffer [89 mm tris(hydroxymethyl)aminomethane base, 89 mm boric acid, and 2.5 mm disodium ethylenediaminetetraacetic acid] at 150 V with a vertical slab apparatus (Hoefer Scientific). The gels were then immersed in an aqueous solution of ethidium bromide (10 μg/ml), stained for 15 min, and photographed under ultraviolet illumination. (A) pWR53 plasmid of 60 Mdal which controls sucrose fermentation; (B) plasmid DNA isolated from Y. pseudotuberculosis strain III; (C) plasmid DNA isolated from Y. enterocolitica strain Y7P; (D) plasmid DNA from a avirulent derivative of Y7P which contains only a 36-Mdal plasmid.*

With the finding of a plasmid in *Y. pseudotuberculosis* similar in size to pWR42 of *Y. enterocolitica* Y7P, we next tested strain III for its calcium dependency when grown at 37°C on magnesium oxalate agar (7). A single colony of strain III was grown overnight at 26°C in broth and diluted in saline to a concentration of 10^4 cells per ml. Then, 10-μl samples were plated in duplicate on magnesium oxalate agar for incubation at either 26 or 37°C. After overnight incubation, the growth of strain III was severely inhibited at 37°C but not 26°C, indicating calcium dependency and hence expression of the V and W antigens (7). Three clones of strain III, recovered at 37°C as derivatives resistant to growth inhibition on magnesium oxalate agar, were examined for their invasive properties and the presence of pWR43. All failed to provoke a positive Sereny test for keratoconjunctivitis and had lost the pWR43. Thus, it is apparent that this virulent strain of *Y. pseudotuberculosis* is similar to *Y. enterocolitica* in that it contains a plasmid associated with pathogenicity and production of the V and W antigens.

We investigated further the similarity of the DNA from these 42-Mdal plasmids of *Y. pseudotuberculosis* and *Y. enterocolitica* by comparing the fragmentation pattern that results after their digestion by restriction endonucleases (11). If the specific sites of restriction enzyme cleavage occur at the same locations in both plasmid molecules, such enzyme treatment will produce a set of fragments from each plasmid that yield

an identical banding pattern on agarose gels. *Y. enterocolitica* strain WA was used for this comparison because this strain contains only the 42-Mdal plasmid (pWR41) and lacks the cryptic 36-Mdal plasmid found in strain Y7P which would complicate the restriction endonuclease digestion pattern (Fig. 2).

Treatment of DNA from both plasmids with the restriction enzyme *Bam*HI yields some fragments that are the same size, but in general the fragment banding pattern is distinct for the two plasmids (Fig. 3). Similarly, *Eco*RI restriction enzyme digestion of these plasmids yields patterns which are different. Even though the plasmids from *Y. pseudotuberculosis* and *Y. enterocolitica* appear similar in size and function, the locations of the specific cleavage sites of the restriction endonuclease are not found at the same positions in the plasmid molecules. Therefore, there are differences in the plasmids at the molecular level.

The present study extends our previous findings on plasmid-associated virulence determinants of *Yersinea*. Although Vwa plasmids from both *Y. enterocolitica* and *Y. pseudotuberculosis* share functions related to the V and W virulence antigens and can be of similar molecular size, the molecular distinctions that were revealed between them by restriction endonuclease analyses indicate a divergence in the microevolution of Vwa plasmids.

We thank Ira Melman for providing strains and Roland Constantineau for technical assistance.

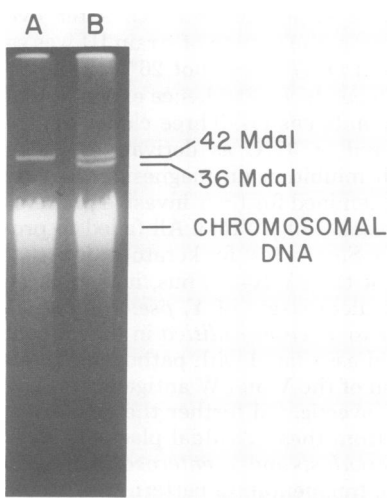

FIG. 2. *Electrophoresis of plasmid DNA from Y. enterocolitica strains WA and Y7P. (A) Strain WA contains a single plasmid (pWR41) which is 42 Mdal in size and has Vwa properties. (B) Strain Y7P contains two plasmids, pWR42 and pWR36, which are 42 and 36 Mdal in size; pWR42 has Vwa-associated functions; pWR36 is cryptic in function.*

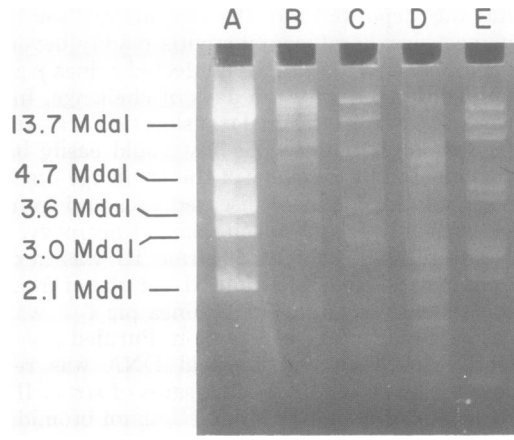

FIG. 3. *Comparison of the banding patterns that are obtained when the plasmids from Y. pseudotuberculosis and Y. enterocolitica are digested with restriction endonucleases and electrophoresed on vertical 0.7% agarose gels. The plasmid DNA was purified and digested with either restriction endonucleases EcoRI or BamHI (New England Bio Labs) before electrophoresis for 20 h at 15 V (constant voltage). The DNA in lane B was only partially digested by BamHI. (A) EcoRI digestion of λ DNA. Sizes of the λ fragments are indicated to provide an estimate of the size of the other fragments. (B) BamHI digestion of pWR43 DNA isolated from Y. pseudotuberculosis strain III. (C) BamHI digestion of pWR41 DNA isolated from Y. enterocolitica strain WA. (D) EcoRI digestion of pWR43 DNA isolated from Y. pseudotuberculosis strain III. (E) EcoRI digestion of pWR41 DNA isolated from Y. enterocolitica strain WA.*

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